Control of Fidelity by the Herpes Simplex Virus Type 1 DNA Polymerase-Associated Exonuclease

Honors Research Thesis

Presented in Partial Fulfillment of the Requirements for graduation "with Honors Research distinction in Molecular Genetics" in the undergraduate colleges of The Ohio State University

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May 2013

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Abstract

DNA replication is a mechanism used to copy and maintain genes during cellular division. As DNA polymerase is copying DNA, mistakes are often made. A combination of this and other environmental factors (UV light, carcinogens) contribute to a high potential for mutations. Thus, faithful replication of DNA (fidelity) is crucial to the viability of a cell. In multicellular organisms, poor DNA replication fidelity can lead to the accumulation of mutations, resulting in deregulated growth and cancer. An important component to the fidelity of DNA polymerases is the associated 3' to 5' exonuclease (exo) activity, which is controlled by a dynamic switching mechanism. This exo activity not only serves to repair mismatched DNA, it also facilitates a cyclic transfer of the elongating DNA strand between the exo site and polymerizing site when the polymerase runs into "road blocks" such as misincorporated nucleotides, abasic sites, or DNA cross-links. Wild type polymerases deal with these "road blocks" by activating the switch behavior to prevent DNA extension until the barrier is removed. Our laboratory studies the herpes simplex virus (HSV) DNA polymerase (pol) as a model enzyme. It has been shown in some HSV pols that a lack of exo activity results in lethal phenotypes. We hypothesize that the switch behavior, rather than the presence of exo activity, is responsible for preserving the quality of synthesized DNA. We believe that this study can lead to the identification of a new target for development of anti-viral drugs and provide a means to screen for compounds that block pol switching and/or exo activity.

General Background

The central dogma of biology depends on a cell's ability to maintain, retrieve, and translate genetic instructions. The organism's genome, is not only used to preserve the viability of an organism, but also allows the information to be passed onto its offspring in a highly preserved form. This principle determines the fitness of an individual within its population. However, in order to maintain the essential genetic information, organisms must possess a means to efficiently and accurately copy their genomes such that all essential genes are passed onto the next generation. This process is achieved by DNA replication.

An organism's genetic information is stored in a chemical from via deoxyribonucleic acids (DNA). Thus, in order to replicate such information, a cell uses a single stranded DNA template to copy its code (or sequence) into a complementary DNA sequence. Eukaryotes have double-stranded DNA and, thus, possess a mechanism to unwind and split the interacting strands such that each strand can act as a template for replication. Each template contains a combination of nucleotides, each of which contains a nitrogenous base, and five-carbon ring sugar, and one or more phosphate groups. There are four different nucleotides, divided into two different groups (separated based on the chemical structures of the bases). The first group contains the purines: adenine (A) and guanine (G). The second group contains pyrimidines: thymine (T) and cytosine (C). As a DNA strand complementary to the template strand is being synthesized, an "A" will always pair with a "T" and a "G" will always pair with a "C." This strict pairing rule allows the genome to be replicated accurately with very few mistakes.

Polymerization

Replication of the genome is primarily carried out by DNA-dependent DNA polymerase. DNA polymerase (pol) adds nucleotides to the free 3'-hydroxyl end of a growing DNA strand. However, it alone cannot initiate DNA replication. Replication origins, specialized sequences within a chromosome, regulate DNA replication by controlling its initiation (1). With the proper assembly of multiple initiation factors onto the origin, the precise onset of replication will occur in the form of a replisome (2). This molecular machinery coordinates DNA replication through the interaction of multiple proteins such as DNA pol, ligase, helicase, primase, etc. (2). DNA pol requires a pre-existing primer (generally RNA) with a free 3' end to begin replication. The primer is synthesized complementary to the template strand by the DNA primase associated with the replisome. The primer is then extended by the DNA pol in the 5'-3' direction.

Double-stranded DNA replication initiation results in a replication fork, an active region of polymerization (3). However, because the DNA strands are anti-parallel and pol can extend only in the 5'- 3' direction, the fork develops an asymmetrical structure in which one strand (the leading strand) is extended continuously from the primer (3,4). This contrasts with synthesis of the other (lagging) strand. In order for the fork to synthesize both strands at the same rate, lagging strand synthesis involves a discontinuous mode of extension in which small lengths of DNA strands call Okazaki fragments are formed, each originating from a free 3' end of a primer (3, 4).

In addition to this 5'-3' polymerase activity, some DNA pols also possess an associated 3'-5' exonuclease activity. Unlike the polymerase activity, which extends DNA at the 3' end, exonuclease activity can remove nucleotides at the 3' end in a competing reaction.

In order for DNA to be synthesized in an accurate manner, DNA pol must discriminate between nucleotides such that during the incorporation stage, the appropriate complementary deoxyribonucleotide triphosphate (dNTP) is added the free 3' end. This nucleotide selectivity is achieved by a combination of mechanisms. The first is the ability of the DNA pol to discriminate between dNTPs and their more abundant ribonucleotide (rNTP) counterparts based on the 2'- hydroxyl group located on the sugar of rNTPs. The pol provides a steric block in its active site, which prevents the binding of nucleotides containing a 2'-hydroxyl group on the sugar ring (5). Stable hydrogen bonding interactions between two properly paired bases also contributes to the accuracy of DNA pol, using it as a criteria for continued replication (6). In addition, evidence has supported the idea that nucleotide selectivity also depends in shape of the complementing base pairs. Only the correct base pairs are capable of fitting into the nascent base-pair-binding pocket without steric interferences (7). It is important to note that DNA pol cannot carry out DNA replication alone. It must coordinate with a multitude of other proteins in the replisome.

Proofreading

The lack of DNA pol proofreading can be threatening to the organism. Proofreading functions of a polyermase is often associated with the removal of

mismatched base pairs. The 3'-5' exonuclease activity allows polymerases to remove misincorporated nucleotides, ensuring high-fidelity DNA synthesis (8). The 3'- 5' exonuclease activity catalyzes the excision of a nucleoside monophosphate (dNMP) from the DNA termini. During excision, the hydrolysis of nucleotide occurs in a distributive manner, such that the processed DNA can return back to the elongation site immediately after error correction (8).

However, proofreading is involved in more than just mismatch excision. Proofreading is also required for optimal polymerase activity (9). Mutational analysis in T4 bacteriophage DNA pol by replacing the catalytically essential aspartate residue for an alanine or glutamine showed a reduction of 3'-5' exonuclease activity by three to four orders of magnitude. The exonuclease reduction was also paired with a decline in DNA synthesis by two to three folds (9, 10). This result is due to the inability for the mutant DNA pol to remove terminal mismatches, thus blocking further primer extension (9). Proofreading also prevents excessive strand displacement synthesis and aids in the maturation of Okazaki fragments during lagging strand synthesis (11). Regulated strand displacement synthesis by DNA polymerase leaves a ligatable nick on Okazaki fragments. Unregulated strand displacement synthesis, on the other hand, produces long 5' flaps that, if not processed correctly, can increase opportunities for misalignment mutations that are lethal (11).

The mechanism of DNA processing in Okazaki fragment maturation is often associated with Flap-endonuclease 1 (Fen-1), which cleaves short 5' flaps created by strand displacement (12). This cleavage function is proposed to be redundant to 3'-

5’ exonuclease and coordinates with the activities of DNA polymerase and ligase to ensure proper DNA synthesis during lagging strand synthesis (13,14). Exonuclease mediates the degradation of a displacing strand at the 3’ end, allowing for the 5’ end of the displaced strand to re-associate with the template and re-form a ligatable nick (Figure. 1).

Fidelity

As previously mentioned, organisms must efficiently and faithfully replicate their genomes to maintain its genomic identity as well as overall viability. Generally, major replicative DNA pols exhibit high fidelity with an *in vivo* mutation rate estimated to be lower than 1×10^{-9}, i.e., less than one error for every billion (or more) nucleotides incorporated (4). Spontaneous mutation rates are approximately 5×10^{-10} for bacterial systems and approximately 5×10^{-11} for mammalian systems (15). Such low rates are the outcome of multiple pathways that act in parallel and consecutively with another. There are three major steps in the fidelity of DNA replication: nucleotide selectivity of DNA pol, proofreading of replication errors by the pol-associated exonuclease activity, and post-replication DNA repair mechanisms.

The first of these processes, nucleotide selectivity to create the correct complementary base pair, has the greatest impact on overall fidelity of DNA replication. The active sites of DNA pols at the site of elongation form the correct conformation that favors the correct nucleotide to fit and pair with the template. This highly selective structure of DNA pols discourages the insertion of mismatched nucleotides that possess a different geometry (7). In particular, the steric clashes that occur from incorrect base pairing in the active site of DNA pol significantly

reduces incorrect dNTP-binding affinity. This, subsequently, hinders the conformational changes needed for catalysis of polymerization. Thus, nucleotide selectivity prevents most errors from forming, contributing to 10^{-4} to 10^{-5} errors per insertion event (15).

In the event that a mismatched nucleotide is inserted, proofreading by either an intrinsic or extrinsic 3'- 5' exonulease activity can excise the error, immediately allowing for the appropriate nucleotide to be inserted in its place (16). Once an incorrect dNTP is incorporated into the elongating strand of DNA, the mismatched primer terminus is poorly extended compared to a properly matched one (4, 9, 16,). The delay in extension caused by the mismatch allows the aberrant 3' terminus to move to the 3'-5' exonuclease site for excision. Proofreading of misinserted bases by 3'-5' exonuclease confers additional fidelity (10^{-2} errors per base pair) (15).

Post-replication DNA repair mechanisms such as translesion synthesis (TLS) by low- fidelity pols and mismatch repair (MMR) are both fidelity systems that further ensure the maintenance of genetic information by correcting errors that have escaped the exonuclease (16). These pathways account for an error rate of 10^{-3} errors per base pair (15). Furthermore, some accessory proteins, like polymerase clamps or single-stranded DNA-binding proteins, can also have an effect, although small, on fidelity (4). Thus, the combination of these mechanisms to overall fidelity account for a mutation rate of approximately 1×10^{-10}. The precise rate of mutation, however, depends on multiple conditions. However, regardless of the exact rate of mutation, reduction in any of the mechanisms will lead to an increase in mutation rates. Higher mutation rates can potentially be the cause of lethality in some

organisms, due to genetic instability or lead to cancers in eukaryotic systems (16, 17).

Despite low rates of replication errors, polymerases are not 100% faithful. In fact, mutations can also generate selective advantages, ultimately creating genetic diversity and variation within a population. Imposed by environmental challenges, genetic changes in make- up are often necessary for the successful adaptation of organisms. However, it is still important to limit spontaneous mutation frequencies to prevent disruptions of crucial cellular processes (18).

DNA Polymerases

Structural and functional studies have identified close relationships between several DNA-dependent DNA pols found in distantly related organisms. Based on sequence homologies and resolved protein structures, these DNA pols are classified into seven different families: A, B, C, D, X, Y, and RT (19). Most eukaryotic DNA pols (α, δ, ζ and ε), viral DNA pols, and some bacteriophage pols are considered to belong to the B type pol classification. Family B pols are multi-subunit enzymes that carry out processive replication of DNA (19,20). With the association to a processivity factor, members of this family of enzymes participate in both leading and lagging strand synthesis. In addition to the 5'-3' polymerase activity, these pols generally also possess a 3'-5' exonuclease activity.

Even though pols from different families are structurally dissimilar, there are several features common to most pols. In general, pols tend to fold in a conformation resembling a right hand with domains designated to the palms, fingers, thumb, and pinky (21,22). The fingers and palm form the polymerase active

site, the pinky resembles the exo site, and the thumb functions in a mechanism called kinetic partitioning (discussed below). In addition to similar architecture, there are six highly conserved regions that have been identified among eukaryotic, prokaryotic, and viral pols (22). Furthermore, the relative positions of these sites to one another are conserved as well.

Epidemiology and Pathology of HSV

Herpes simplex virus type 1 (HSV-1) DNA pol is the target for many anti-viral therapeutics. Eight of 100 of the family of *Herpesviridae* are routinely known to infect humans: HSV-1 and 2, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus, human herpesvirus 6 and 7, and Kaposi's sarcoma virus (human herpesvirus 8) (23,24). Herpesviruses are a leading cause of human viral diseases, second only the common cold viruses. HSV is responsible for a contagious infection that affects nearly 60%- 95% of adults worldwide (24). All herpes viruses are enveloped, double-stranded DNA viruses. In addition, all encode a DNA pol with 3'- 5' exo activity. HSV-1 also encodes more than 84 polypeptides, with the DNA pol being one of those polypeptides. HSV-1 and 2 are responsible for a wide variety of diseases that greatly affect the quality of life for infected individuals. In addition, although not usually life- threatening, HSV infections can be the cause of significant mortality in immunocompromised patients as well as in infants (25).

With viral infections contributing to significant rates of mortality and morbidity, there is an absolute need for promising anti-viral drugs and therapies. Because DNA pol is essential for virus viability, it is an excellent target for novel anti-viral drugs. Targeting viral proteins, such as HSV DNA pol, has an advantage of

minimizing host side effects (25). However, viral protein-targeted drugs can act as a selective pressure against the virus and may result in drug-resistant viral strains. Such is the case with acyclovir, a nucleoside analogue that is phosphorylated by viral thymidine kinase and, once incorporated, inhibits dNTP incorporation into the elongating viral DNA, causing chain termination (26). The development of drug-resistant strains have been observed in immunocompromised individuals, such as patients infected with human immunodeficiency virus (HIV).

HSV-1 Polymerase

The HSV-1 DNA pol is a 1235 amino acid protein encoded by the UL30 gene (27). It exists as a stable heterodimer with the UL42 gene product, a processivity factor, which aids in polymerization. The HSV-1 DNA pol exhibits sequence homology to other viral and cellular B-type DNA polymerases. The crystal structure of the HSV-1 DNA pol was recently resolved and shows a similarity in the arrangement of the domains in a right hand shaped (28). Like most type B DNA pols, HSV-1 DNA pol has both polymerizing and exonuclease activities. Most of the C-terminal half constitutes the polymerization domain and contains conserved sites essential for polymerase activity. The intrinsic 3'-5' exonuclease activity of the HSV-1 DNA pol is associated with the N-terminal half of the protein encoded by UL30. Three sequence motifs are present and are homologous to the exonuclease sequence motifs (called exo I, II, and III) of *E. coli* DNA pol I as well as many other DNA pols (27). This reveals the highly conserved nature identified within the exonuclease regions. Exo site I is known to contain two of the catalytic residues, which coordinate divalent cations, such as Mg^{2+}, required for cleavage (29). Site II

may contain another catalytic glutamate residue. Site III does not directly contribute to the catalytic activity of the intrinsic exonuclease, however, it does contribute to the 3-D structure of the polymerase.

Kinetic Partitioning

Kinetic Partitioning describes a phenomenon in which two competing reaction routes take place to produce a particular biological output (30). In the case of DNA extension and fidelity, the competing routes of a DNA pol is the association of the elongating strand between the active polymerizing site or the exonuclease site. Typically, it is thermodynamically favored for DNA to bind onto the pol site. However, when a mismatch incorporation occurs, the polymerase undergoes a process of idling turnover, which is characterized by successive rounds of nucleotide excision and incorporation by DNA pol. This idling behavior ultimately increases overall DNA replication fidelity because it prevents extension through irregular DNA regions. These barriers to DNA replication can be caused by mismatched base pairs created by mistakes in replication or by DNA lesions, which include abasic (AP) sites, oxidative lesions, or bulky lesions (31). Such instances of DNA damage can be caused by chemical or environmental insults to DNA (32).

One of the major functions of DNA pol proofreading relies on the pol's ability to recognize a misincorporation event (9). The end of the elongating DNA strand is able to move from the polymerase to the exonuclease site if a kinetic barrier to extension is encountered. In general, the binding of DNA to the polymerase site of the DNA pol is thermodynamically more favorable than the binding to an exonuclease site (9). Thus, under normal conditions, it is expected that DNA will be

more likely bound in the active site of DNA pol, undergoing elongation. However, under the pressure of a kinetic block, the template stand can be switched onto the exonuclease site. The rate of a conformational change to initiate exonuclease activity with proper base pairing is slow due to the fact that the binding of DNA to the polymerase site is more favored. But since the extension of a mismatched primer terminus is even slower than the rate of DNA switching, kinetic barriers will usually favor proofreading over the extension of a mismatch (9). After the proper excision of a mismatched nucleotide, the trimmed end can now be returned to polymerase site to finish replication with the correct dNTP. Due to the fact that exonuclease competes for the binding of the 3' terminus strand with the polymerase site, a reduction in polymerase speed and/or efficacy is a caveat to high-fidelity modes of replication. However, pre-steady state kinetic experiments have shown that the ability if the polymerase to switch between the polyermase and exonuclease sites when necessary is critical to the polymerases' ability to maintain an active and rapid rate of polymerization with high-fidelity replication (31).

Studies with mutations in the three conserved exonuclease motifs (exo I, II, and III) have been used to examine the role of 3'-5' exonuclease activity in maintaining the fidelity of HSV-1 DNA pol (19). Mutations on all three of the exo sites lead to a reduction in exonuclease activity. However, mutations in exo I and II led to lethal phenotypes (30). Catalytic exo I mutants showed a complete lack of exonuclease activity, but maintained a wild-type-like polymerase activity that was even greater than WT activity. Exo II pol mutants, on the other hand, possessed defects in both pol and exonuclease activity. Exo III mutants were able to maintain

extension at or close to WT levels, but displayed a mutator phenotype (31). This study involving exonuclease-deficient mutants presents a curious case about the role involved with the exonuclease. The ability for exo III to replicate DNA, despite the absence of exonuclease activity and the presence of WT polymerase activity, compared with the exo I mutant's lethal phenotype, even with a near-wild-type polymerase activity, suggests that the region included in the exo I site has a more intricate role in replication (31). Perhaps it may even participate in the dynamic switching mechanism of kinetic partitioning.

Research Project

Studies of exo activity and mechanisms associated with kinetic partitioning reveal a complex set of events by which DNA pol can maintain fidelity. As discussed above, fidelity is not simply mediated by exo activity, but, perhaps, multiple parallel mechanisms that ensure faithfulness as well as effectiveness of DNA extension by DNA pol. My project goals were to determine the roles of kinetic partitioning and exo activity in the overall function of DNA pols. In order to determine possible new targets for the development of anti-viral drugs, an additional goal was to provide a means to screen for such compounds via high-throughput screenings.

The overarching hypothesis guiding my studies was that when a polymerase encounters a kinetic barrier, defects in the switching behavior of polymerases contribute to the overall quantity and quality of synthesized DNA. If true, this may explain the previous observation of a lethal phenotype of exo I pol mutants. My project has been designed to focus on two specific aims:

1. To create mutants that will allow us to establish a relationship between pol switch behavior, exo activity, and high-fidelity DNA synthesis.
2. To develop fluorescent assays capable of quantifying the switch behavior and exo activities of mutant polymerases.

Materials and Methods

Mammalian Cell culture. Baby hamster kidney (BHK) cells were maintained in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 7.5% fetal bovine serum (FBS, Atlanta Biologics, Atlanta, GA), 100 units/mL penicillin, 100 μg/mL streptomycin sulfate, 0.03% glutamine, and 0.075% sodium bicarbonate. .

Plasmid Construction. The plasmids used in the following procedures includes pUC19 and pcDNA3.1. Pol genes were inserted into these vectors by utilizing the restriction sites of XbaI and HindIII. These sites were located 51 nucleotides (nt) upstream of the open reading frame (ORF) of the inserted pol gene and 118nt downstream of the ORF, respectively. Vectors were named by stating the plasmid name followed by the pol gene. For example, a wild- type pol gene in a pUC19 vector will be called pUC19-WT, whereas a mutant D368A pol mutant gene in a pUC19 vector will be called pUC19-D368A. The same naming method would be applied for the pcDNA3.1 plasmid.

The plasmid pDsRed with a HSV origin was used in a complementation assay (described below). The HSV origin, which was 560 bp, was inserted via a BamHI restriction site into the pDsRed plasmid (ClonTech, Mountainview, CA).

Site-Directed Mutagenesis of Expression Plasmid (Procedure I). Polymerase chain reactions (PCR) were carried out in a 100 μL reaction volume and began with a hot start at 94°C for 10 min, prior to the addition of *Pfu Turbo* DNA polymerase (Agilent Technologies, Santa Clara, CA). Mutagenic primers were designed to follow the guidelines specified by the QuikChange II XL Site-Directed

Mutagenesis Kit from Agilent (La Jolla, CA). PCR reactions contained 250 μM dNTP, 150 μM forward primer, 150 μM reverse primer, 35 ng pcDNA pol dsDNA template, 10% dimethyl sulfoxide (DMSO), 1 unit of Pfu DNA polymerase, and 1× Clone Pfu DNA polyermase reaction buffer from Agilent Technologies, which consisted of 20 mM Tris-HCl (pH 8.2), 2 mM $MgSO_4$, 10 mM KCL, 10 mM $(NH_4)_2SO_4$, 0.1% Triton®, and100 μg/mL nuclease-free bovine serum albumin (BSA). PCR primer combinations as well as amplification conditions are specified in Table 1. Specific PCR primer sequences could be found in Table 2.

The PCR products were precipitated with ethanol and resuspended in 50 μL of 1×NEBuffer #4 (New England Biolabs, Ipswich, MA), containing 50 mM potassium acetate, 20 mM Tris- acetate, 10 mM magnesium acetate, 1 mM dithiothreitol (DTT) and 10 unit of Dpn1 restriction enzyme. The reaction was incubated at 37°C for 1 hour to digest the parental plasmid. An aliquot (4 μL) of Dpn1-treated DNA was transformed into XL-1 Blue supercompetent *Escherichia coli* cells that were provided by the QuikChange II XL Site-Directed Mutagenesis Kit.

Based on blue-white color screening, the expected colonies were selected. DNA was isolated and sequenced to verify the desired mutation.

Overlap Extension PCR Mutagenesis (Procedure II). A schematic for the overlap extension PCR method can be found in Figure 2. Briefly, 80 ng of parental plasmid pUCpol was linearized with Xba1 and used as a template in the "left" and "right" PCR reaction. Primers were selected with the aid of the Oligo7 software (Molecular Biology Insights, Inc., Cascade, CO). Primer sequences can be found on Table 3. The sequence of the reverse primer for the "left" PCR reaction is

complementary to all or a portion of the forward primer for "right" PCR reaction. Both mutatgenic primers contain a mismatched base to the target sequence. The "left" and "right" PCR reactions were carried out in a total volume of 50 μL reactions containing 1× Clone Pfu DNA polyermase reaction buffer, 250 μM dNTP, 500 μM forward primer, 500 μM reverse primer, 50 ng/ μL template DNA, 10% DMSO, and 1 unit of Pfu DNA polymerase. PCR reactions were initiated with a hot start at 94°C for 10 min. This was followed by 30 PCR cycles of denaturing for 2 min at 94°C , annealing for 2 min at 62°C , and extension for 4 min at 68°C. PCR products were purified with the QIAquick PCR Purification Kit from Qiagen (Valencia, CA) for next step.

Equal volumes of the "left" and "right" PCR reactions were combined and used as a template for the next step. Reactions were carried out in a 100 μL reaction volume under the conditions previously described for the left and right reactions. The PCR product was purified, digested with NheI and SacI, separated by agarose gel electrophoresis, and the fragment NheI-SacI fragment was purified with the QIAquick Gel Extraction Kit from Qiagen (Valencia, CA). The gel-purified fragment was cloned into a similarly purified pUCpol (wild-type) plasmid from which the NheI-SacI fragment had been removed. The desired mutation and the entire sequence of the pol gene insert was confirmed by DNA sequencing. The pol gene containing the desired mutation was excised by XbaI and HindIII from the pUC plasmid and inserted into the XbaI and HindIII sites of pcDNA3.1, a mammalian cell expression vector under the control of a cytomegalovirus (CMV) promoter for

further studies. The entire pol gene was again sequenced to confirm that no other mutations were present.

Origin-Dependent DNA Amplification Assay. BHK cells (8×10^5 /60 mm dish) were seeded in antibiotic-free medium containing 7.5% FBS, 0.225% sodium bicarbonate, and incubated at 37°C in a 5% CO_2 atmosphere. After overnight growth to achieve approximately 75-80% confluency, liposome-directed transfection was carried out in OPTI-MEM reduced serum medium (Gibco, Grand Island, NY). For each 60 mm plate, a total of 3.6 μg of DNA was used for transfection, which consisted of 1.2 μg of pDsRedori or parental DsRed plasmid, and 2.4 μg of pcDNA containing the HSV wild type or mutant pol gene. Transfection with only pCU19-WT was used as the negative control. DNA was diluted to a total volume of 150 μL with Opti-MEM (Invitrogen, Carlsbad, CA) and mixed with 150 μL of a 1:20 dilution of lipofectamine 200 (Invitrogen, Carlsbad, CA) in Opti-MEM according to the manufacturer's instructions. After 20 min, these mixtures were brought to 1.5 mL with Opti-MEM and added to the appropriate 60 mm plate. After 4 hours, the transfection medium was replaced with DMEM containing 5% FBS and incubated for an additional 20 hours at 37°C. At 24 hours post-transfection, cells were infected with HSV pol null mutant (ΔS) virus at a multiplicity of infection (M.O.I.) of 5 plaque forming units (PFU)/cell. Cells were incubated as before and harvested at 18 hours post-infection (p.i.). Cells were collected into the medium by scraping and pelleted by centrifugation for 5 min at 700 x g. Cell pellets were washed twice with PBS without Ca^{2+} and pellets were frozen at -80°C. The DNA from frozen cells was

isolated using the DNAeasy Tissue kit from Qiagen; following the manufacturer's instruction. DNA concentrations were determined by spectrophotometric readings.

Southern blot hybridization. The template for making the hybridization probe was produced by PCR (primers can be found on Table 4). DNA probes were made with Rediprime II random priming labeling system from (GE Healthcare, Piscataway, NJ) based on the manufacturer's instructions. Probe containing 2.5-4.7 x 10^7 dpm was used for southern blot hybridization.

Southern blot. DNA (2 μg) from each sample was digested with BamHI and BamHI/DpnI in 50 mM potassium acetate, 20 mM Tris- acetate, 10 mM magnesium acetate, and 1 mM DTT. The digested DNA product was subjected to electrophoresis in a 1% agarose gel containing 0.1% ethidium bromide (EtBr) and visualized with a UV transluminator (Bio-Rad). The DNA within the gel was depurinated in 0.25M HCl, denaturated with 0.5M NaOH and 1.5M NaCl, and the gel was then neutralized in 1 M Tris, pH 7.5 and 1.5 M NaCL. DNA was transferred to a Hybond-N nylon membrane (Amersham Biosciences, Piscataway, NJ) by capillary transfer. Membranes were baked at 80°C for 2 hours to fix that DNA and were pre-hybridized in a hybridization buffer containing 450 mM NaCl, 45 mM sodium citrate, 0.04% heat inactivated BSA, 0.04% polyvinyl pyrrolidone-4000 MW, and 0.04% Ficoll with a pH of 7 for 4 hrs at 68°C. The buffer was changed and hybridization was carried out in at 68°C overnight. The membrane was washed twice in a 2xSSC solution (1X SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0) at 68°C for 15 minutes. It was then washed again with 1x SSC at 68°C for 15 minutes before exposure to a storage phosphor screen.

HSV-1 Polymerase activity Assay. Reactions were carried out in 5 mM EDTA, 140 mM Tris-HCl (pH 8.0), 2.8 mg/mL BSA, 2 mM DTT, 4% Glycerol, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, 5 nCi/μL, 2.9 μg activated calf thymus DNA, and 12.5 mM KCl. This reaction mix was incubated with 5 μL of diluted pol/UL42 at 37°C for 5 min before initiation of the reaction with 6 mM $MgCl_2$. Pol/UL42 dilutions were carried out in a buffer containing 0.1 M KCl, 0.01 M Tris-HCl (pH 8.0), 10% glycerol, and 1 mg/mL heat activated BSA. Reactions were stopped by spotting 70 μL of the reaction mix onto a 2.3 cm Whatman filter from Whatman International Ltd. (Springfield Mill, UK). The filters were then immersed in ice cold 5% TCA and 0.5% sodium pyrophosphate (Na PPi) for 15 min, twice, to precipitate DNA. Filters were then washed two times with 95% ethanol for 2 min. Filters were dried, added to scintillation fluid, and analyzed for counts through a LS 6000IC Beckman liquid scintilation counter.

Fluorescence Assay Probe Preparation. Probe sequences were designed to form a hairpin conformation to create either a matched or mismatched base pair at the primer/template (P/T) interface followed by a cytosine tail (Figure 3). Probes were synthesized, tagged at the 5'- end to a Fluorescein (FAM) fluorescent dye, and purified with high-performance liquid chromatography by Integrated DNA Technologies (Coralville, IA).

Fluorescence Assay. The fluorescent-tagged DNA probes (200nM) were added to 125 mM KCl, 120 mM Tris, 10 mM DTT, 200 μg/mL BSA, 5 mM EDTA, 500 μM dTTP, and 500 μM dGTP. Initially, 35 μL of the DNA probe reaction mixture was brought to a total volume of 52.5 μL with 10 μL of DNA pol and H_2O. This reaction

volume was prepared per variable that was being examined (e.g. time). The mixture was then incubated at 37°C for 5 min to establish pre- steady state conditions. Reactions were carried out at 37°C and initiated with 17.5 μL of $MgCl_2$. Reactions were stopped with 14 μL of 0.25M EDTA.

Results and Discussion

Aim 1: To create pol gene mutants to examine the relationship between pol switch behavior and exo activity.

HSV-1 pol gene mutations within conserved exo sites were created by site-directed mutagenesis. Targeted domains included codons within the conserved exo I and exo III domains. Within the exo I domain, the glutamate codon at the 368th position of the pol open reading frame (ORF) was changed to an alanine (D368A). Two mutations were made in the exo III domain—a tyrosine to histidine substitution at the 577th position (Y577H) and a glutamate to alanine substitution at the 581st position (D581A) of the pol ORF. The two exo III mutations were also combined to form a double mutant called YDmut.

Mutations were generated by two different methods of PCR mutagenesis. The first involved a conventional PCR method in which the circular expression plasmid containing the wild-type pol gene (pcDNA3.1-WT, 9184nt) was used as a template for a set of two overlapping mutagenic primers to be extended by the high fidelity polymerase, PfuI, to form the high molecular weight products that were transformed directly into *E. coli*. These optimizing PCR conditions were initially tested with the amplification of pU19-WT where products of full-length size (~6.4Kbp) were generated (Figure 4). Complete sequencing of the pol gene insert confirmed that this method yielded the Y577H mutant pol gene. However, after numerous attempts to optimize PCR conditions (e.g. time, annealing temperature, this method proved ineffective at yielding the D368A mutations (Table 1). There are several possible explanations for this result. It should be noted that the pUC19- pol

plasmid, is quite large, 6492 bp, and the pol gene has a high G+C content (68.3%) (33,34). Most of the conditions tested demonstrated that the mutagenic primers selected were not utilized, suggesting that inefficient annealing had occurred. To increase annealing, the temperature was lowered. However, this utilized the primers without yielding full-length product. The shorter than full-length products were likely due to false priming. This could be a potential explanation for the smaller bands that appeared on the gel in Figure 4. The primer length could not be increased further to allow for higher annealing temperatures, without increasing false priming as predicted by the Oligo 7 program (not shown).

In order to select mutagenic primers more compatible with size and G+C contet of the template, an alternative method was performed (Recombinant PCR). With this method, instead of having to amplify the entire plasmid, a mutation can be created within a specified plasmid fragment. As shown in Figure 2, a specific gel-purified fragment of the pUC19- pol plasmid was subjected to PCR mutagenesis with a wild-type and mutagenic primer to separately amplify sequences to the left and right of the targeted site. After each set of primers has undergone 30 cycles of amplification, the two PCR products were combined and amplified with the WT primers that flanked the target sequence. Figure 5 demonstrates specific amplification of the left and right sides with few products different in size. Likewise, most of the product produced by amplifying the combined products was of the size predicted (1312 bp), suggesting efficient priming and 3' extension.

Unlike what was observed with the previous procedure, the D368A mutation was successfully cloned into the fragment. It was then successfully cloned into the

pCDNA 3.1 mammalian expression vector and the entire sequence of the pol gene was confirmed to contain only this single mutation.

Ability of mutant pol genes to function in an origin-dependent DNA replication assay. Bacterial plasmids cannot generally be amplified in mammalian cells because they do not contain a functional mammalian cell origin of replication. However, plasmids that contain an HSV-1 origin sequences can be amplified in the presence of a functional HSV-1 DNA replisome containing all of the 7 essential DNA replication proteins (35). Thus, an ori-containing plasmid will amplify when wild-type virus is introduced. A modified version of this assay has been used to test the ability of transfected genes to complement plasmid amplification when the virus introduced lacks that gene of interest. The virus used in the following assays was deleted for the pol gene (pol null [ΔS] mutant). The engineered pol exo site mutants within the mammalian expression vector pcDNA3.1 were individually introduced into BHK cells together with an indicator plasmid (pDsRed) containing a functional HSV-1 origin of replication (DsRedori). Total DNA was isolated from the cells following a single cycle of virus replication (18 hr) and amplification of plasmid sequences was determined by Southern blot hybridization to a plasmid-specific DNA probe. Input plasmid of bacterial origin was cleaved with Dpn1, while the amplified plasmids was cleaved with BamHI. Cells transfected with WT pol gene and DsRedori was used as a positive control. As a negative control, plasmid lacking a pol gene (pUC19) and the pDsRedori were introduced but were not infected. In the presence of pol null mutant virus, amplification was observed with the WT pol gene and all of the mutant pol genes when expressed from the expression plasmid.

Surprisingly, amplification pDsRedori also occurred in the presence of the wild-type gene in the pUC19 vector and the pol null mutant. Since the pol gene cloned into the puc19 and pCDNA vectors contains ~51 bp upstream to the ORF, it is likely that these sequences contain sufficient promoter activity to allow it to be transcribed by the mutant virus. In the future, transfection with pcDNA3.1 would be a more appropriate negative control.

The amount of amplification observed in each sample was quantified to assess whether the mutated pol genes could amplify as well as the wild- type. Cells transfected with pUC19-WT pol and DsRedori showed similar fold amplifications as the pcDNA3.1- WT pol transfected cells (Figure 6). However, each of the mutant exo-site pol genes amplified more of the input ori-containing plasmid than did either wild-type pol gene. In viewing the blot, it was apparent that there was less signal in the DpnI digested (input) fragment of the mutant compared to wild-type transfected samples, although the BHK cells were all treated with equal amounts of DNA. If the cells were capable of replicating only a certain amount of DNA, introduction of less DNA could result in greater fold-amplification. Although the exact reason why more DNA is amplified by the mutant pol genes is not known, it is clear that these genes possess no defect in the quantity of DNA that can be replicated in an origin-dependent fashion.

Aim 2: To develop fluorescent assays capable of quantifying the switch behavior and/or exo activities of polymerases.

A fluorescence assay was developed to quantify the exo activity as well as switch behavior in mutant exo-deficient pols relative to WT pols. The assay relies on

fluorescent probes that carry either a match or mismatched base pair at the primer/template interface (Figure 3). These probes act as DNA pol substrates and can be extended from the 3'-end of the probe. Stacked G residues in the extended portion opposite the fluorescent label will interact with the fluorescent marker attached to the 5'-end on the hairpin, ultimately, quenching the fluorescent signal (36). Thus, the quenching of the fluorescent marker depends on the ability of DNA pol to extend beyond the matched or mismatched base pairs. With matched probes, it would be expected that both the WT and the exo-deficient pols would exhibit similar abilities to extend the 3'- end and quench the fluorescence marker. In a mismatched probe, WT pol can repair the mismatched base pair by replacing it with a correctly paired nucleotide and continue extension (and therefore switching) nearly as efficiently as the case with the match probe. On the other hand, exo-deficient mutants will display poorer extension capabilities (i.e. less reduction in fluorescence) since they cannot excise the mismatch.

Preliminary testing of this assay demonstrated its ability to differentiate the WT pol from the exo-deficient mutants. Under conditions of excess enzyme concentration, the assay was first used to determine the amount of time in which the WT pol/UL42 enzyme complex can exhaust the fluorescent substrate. Based on the kinetic profile seen in Figure 7, it was determined that the substrate was exhausted by wild-type pol by 2 min or less, whether it was matched or mismatched.

This two min time frame was then used to compare the WT pol/UL42 complex with the exo-deficient pol/UL42 complexes (Figure 8). The level of fluorescence reduction of the matched probes by the three pols was relatively

similar. However, it should be noted that the WT pol appears to be slightly less efficient in quenching the probes when compared to the exo-deficient mutants. This is likely due to the fact that WT pols experience competition in the switching of the nascent strand between the active and the exo sites. Exo-deficient mutants, on the other hand, lack exo activity and thus do not need to deal with the competition for the binding of the DNA substrate between the two domains (9). In contrast to the results seen with the matched probes, the mismatched probes showed a considerable difference in the level of fluorescence reduction by the three pols. Both the exo I and exo III mutants extended the mismatched probe less efficiently than the WT pol. Furthermore, in this experiment, the exo III mutant was more capable of extending the mismatched probe compared to the exo I mutant. This suggests that the exo III mutant might have more switching activity than the exo I mutant since both mutant pols completely lack exo activity (31). More experiments with precisely the activities among all the enzymes will be necessary to confirm these differences.

When preparing the assay by pre-incubation of the enzyme with its DNA substrate prior to initiating polymerization via the Mg^{2+} catalyst, pre-steady state conditions were approximated. After initiation, it was noticed that two distinct rates of fluorescence reduction could be detected: an earlier faster rate followed by a slower rate, interpreted to be the steady state rate and/or utilization of the substrate (Figure 9).

Because most of the DNA substrate was utilized during the fast rate, conditions were optimized to better characterize the steady-state rates of the enzymes and to determine if differences can be used to differentiate the mutant

compared to wild-type pol activities. Various conditions were tested with a WT pol/UL42 complex according to the reaction set-up described in Materials & Methods. The first variable tested was the enzyme concentration, where the relative pol activities of enzymes were diluted and tested by a traditional assay that relied on incorporation of labeled dTTP into an activated DNA template (ref.). In this assay, dilutions of the WT and mutant pols were incubated and the kinetics of incorporation were determined over a 15 min period at 37°C. Rates were determined by linear regression of a dilution of pol in which DNA amount was not limiting. The rates are shown in Table 5. Based on the wild-type enzyme, one unit of enzyme activity was defined at the activity required for the incorporation of 50 counts per min (cpm) per min of dTTP per μL of enzyme at 37°C.

One goal of the FRET assay was to utilize it to screen for inhibitors of pol-associated exo activity. To be useful, the assay would need to distinguish different amounts of activity, preferably at least a 2-fold difference. We tested the matched fluorescent probes with two concentrations of enzyme (16 and 32 units). It was predicted that reactions with 16 units of enzymatic activity would have a rate of reduction in fluorescence half that seen with 32 units (Figure 10). Reactions rates from this assay show that with matched probes, the reaction rate of a 32 unit reaction was approximately 1.3 times greater than with a 16 unit reaction (Table 6). On the other hand, there did not seem to be very much difference between the 32 and 16 unit reactions with mismatched probes. These results show under the conditions tested, the FRET assay may not be effective in revealing a two-fold difference in activity. One possible explanation for this outcome could be due to the

fact that substrates have already been exhausted in either or even both reactions prior to establishing a steady state rate.

To ensure that DNA was in great excess over enzyme amount, I altered the amount of DNA by using increasing amounts of unlabeled hairpin probe to a constant amount (200 nM) of fluorescent probe. Undiluted probes were compared to reactions with a two-fold as well as a three-fold amount of DNA (Figure 11). By reducing the specific activity of the probe, it was predicted that excess DNA could be established. If so, it was expected at a two-fold reduction in the specific activity of the labeled probe would cause a rate of fluorescence reduction that was approximately two times slower than that with the undiluted labeled probe. However, much like the previous results, the rates of reduction remained relatively constant at all DNA concentrations (Table 11). In addition, it was noted that there was no difference in rates of the two enzyme amounts tested. Thus, neither changes in DNA nor enzyme amount could be detected by the FRET assay.

Table 1. Site-direct Mutagenesis PCR Conditions, Attempts, and Results

Primers	Reaction Conditions	Results Description
D368A1556F28 D368A1554R28	2 Step PCR: 94°C (1 min), 68°C (7 min) Notable Reagents: pUC19, Taq	Primers not used, inefficient annealing
“ ”	2 Step PCR: 94°C (1 min), 68°C (7 min) Notable Reagents: pUC19, Taq, DMSO	Primers not used, inefficient annealing
“ ”	3 Step PCR: 94°C (1 min), 55°C (1 min), 68°C (7 min) Notable Reagents: pUC19, Taq, DMSO	Primers not used, inefficient annealing
“ ”	3 Step PCR: 94°C (1 min), 55°C (1 min), 65°C (7 min) Notable Reagents: pUC19, Taq, DMSO	Primers not used, inefficient annealing
“ ”	3 Step PCR: 94°C (1 min), 55°C (1 min), 68°C (7 min) Notable Reagents: pUC19, Pfu, DMSO	False priming, inefficient annealing
“ ”	3 Step PCR: 94°C (1 min), 55°C (1 min), 65°C (7 min) Notable Reagents: pUC19, Pfu, DMSO	False priming, inefficient annealing
D368A1555F25 D368A1559R25	2 Step PCR: 94°C (1 min), 68°C (7 min) Notable Reagents: pUC19, Pfu, DMSO	Primers not used, inefficient annealing
“ ”	3 Step PCR: 94°C (1 min), 55°C (1 min), 65°C (7 min) Notable Reagents: pUC19, Pfu, DMSO	Primers not used, inefficient annealing
Y577H2187F28 Y577H2181R2	2 Step PCR: 94°C (1 min), 68°C (7 min) Notable Reagents: pUC19, Taq, DMSO	Full amplification of pUC19 was detected, false priming

Table 2. Site-directed Mutagenesis Primers

Primer Name[a]	Primer Sequence[b]
Y567H2187F28	5'-TCGGCGAG**C**ACTGCATACAGGATTCCCT-3'
Y567H2181R25	5'-TGTATGCAGT**G**CTCGCCGATCACC-3'
D368A1556F28	5'-CTCATGTGCTTCG**C**TATCGAATGCAAGG-3'
D368A1554R28	5'-TTGCATTCGATA**G**CGAAGCACATGAGCT-3'
D368A1555F25	5'-GCTCATGTGCTTCG**C**TATCGAATGC-3'
D368A1559R25	5'-CCTTGCATTCGATA**G**CGAAGCACAT-3'

[a.] Primer names were given based on the particular amino acid substitution. This is followed by the site of nucleotide exchange based on a reference point that was 467nt upstream of the ORF. The primers are then designated as either a forward (F) or reverse (R) primer. The last number corresponds to the size of the primer.

[b.] Nucleotides underlined and bolded in the primer sequences point out the specific nucleotide substitution of the mutagenic primer.

Table 3. Overlapping Site-directed Mutagenesis Primers

Primer Name[a]	Primer Sequence[b]
D368A1554F28	5’-AGCTCATGTGCTTCG**C**TATCGAATGCAA-3’
D368A1559R25	5’-CCTTGCATTCGATA**G**CGAAGCACAT-3’
WT1323F25	5’-TCTGCCCGGCCATCAAGAAGTACGA-3’
WT2609R26	5’-GGGTACAGGCTGGCAAAGTCGAACAC-3’

[a.] Primer names were given based on the particular amino acid substitution. This is followed by the site of nucleotide exchange based on a reference point that was 467nt upstream of the ORF. The primers are then designated as either a forward (F) or reverse (R) primer. The last number corresponds to the size of the primer.

[b.] Nucleotides underlined and bolded in the primer sequences point out the specific nucleotide substitution of the mutagenic primer.

Table 4. Southern Blot Probe Preparation Primers.

Primer Name[a]	Primer Sequence
pDsRed24051F24	5’-TTGCCGGATCAAGAGCTACCAACT-3’
pDsRed24468R26	5’-GGACTATAAAGATACCAGGCGTTTCC-3’

[a]. The name of the primer used for pDsRed2 amplification designated the site on the plasmid in which the first nucleotide of the primer anneals to based on a reference point 2576nt upstream of the DsRed2ORF. The primers are designated as either a forward (F) or reverse (R) primer. The last number corresponds to the size of the primer.

Table 5. HSV-1 Polymerase Activity Assay.[a]

Polymerase name/ dilution	Reaction Rate (dpm/min)
WT/ 1:160 dilution	5.94×10^3 dpm/min
D368A/ 1:10 dilution	824 dpm/min
YDmut/ 1:80 dilution	4.89×10^3 dpm/min

[a.] The data from the HSV-1 polymerase activity assay measured the reaction rate of a reaction mixture containing a particular concentration of either WT, D368A, or YDmut pol in the presence of H^3-dTTP. Liquid scintillation counters detected the level of H^3-dTTP incorporated by the pol onto nicked calf thymus DNA.

Table 6. Fluorescence Assay with varying enzyme concentrations.[a]

Pol Activity (in units)/matched or mismatched	Reaction Rate (units/min)
16 units/ matched	1.58±0.17
32 units/ matched	2.08±0.27
16 units/ mismatched	0.935±0.15
32 units/ mismatched	0.988±0.05

[a.] Fluorescence Assays were carried out by using varying enzymatic concentrations exposed to either a fluorescent-tagged probe with a match or mismatch base pair on the primer/template interface (refer to Figure. 3). Extension of the primer/template by the pol with dGTPs will quenched the fluorescent tag at the 5'-end of the probe, therefore, allowing for the detection of the reaction rate of reduction in fluorescence over the 20 min time frame.

Table 7. Fluorescence Assay with varying enzyme concentration and specific activity.

Pol Activity (in units)/ total DNA concentration (nM)[a]	Reaction Rate (arb. units/min)
16 units/ 200nM	4.650±0.403
16 units/ 400nM	4.475±0.191
16 units/ 600nM	4.699±0.550
32 units/ 200nM	4.199±0.886
32 units/ 400nM	4.042±1.147
32 units/ 600nM	4.560±0.434

[a.] To alter specific activity of the marked probes, total DNA concentrations were increased by introducing increasing amounts of unlabeled probes while maintaining labeled probe concentrations at 200nM.

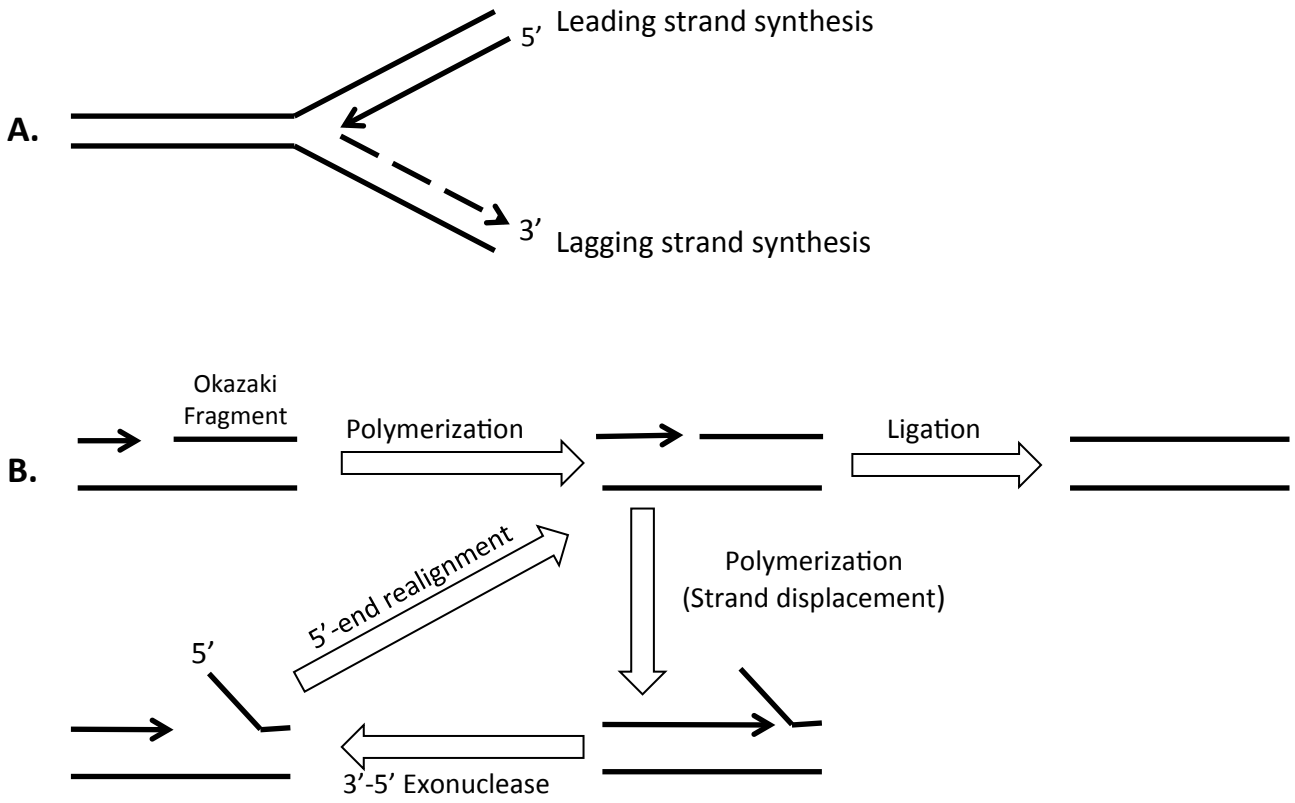


Figure 1. Lagging strand Synthesis. (A) Replication at the replication fork consists of the leading strand and a lagging strand. Since polymerization occurs only in the 5’- 3’ direction, one strand (the leading strand) can be synthesized continuously. However, lagging strand synthesis occurs discontinuously, resulting in Okazaki fragments. (B) During lagging strand synthesis, DNA pol can extend beyond a 3’ nick and lead to strand displacement. The 3’- 5’ exonuclease can resolve this problem by excising excess nucleotides on the displacing strand, and thus allowing the 5’- end of the displaced strand to realign and reform a ligatable nick.

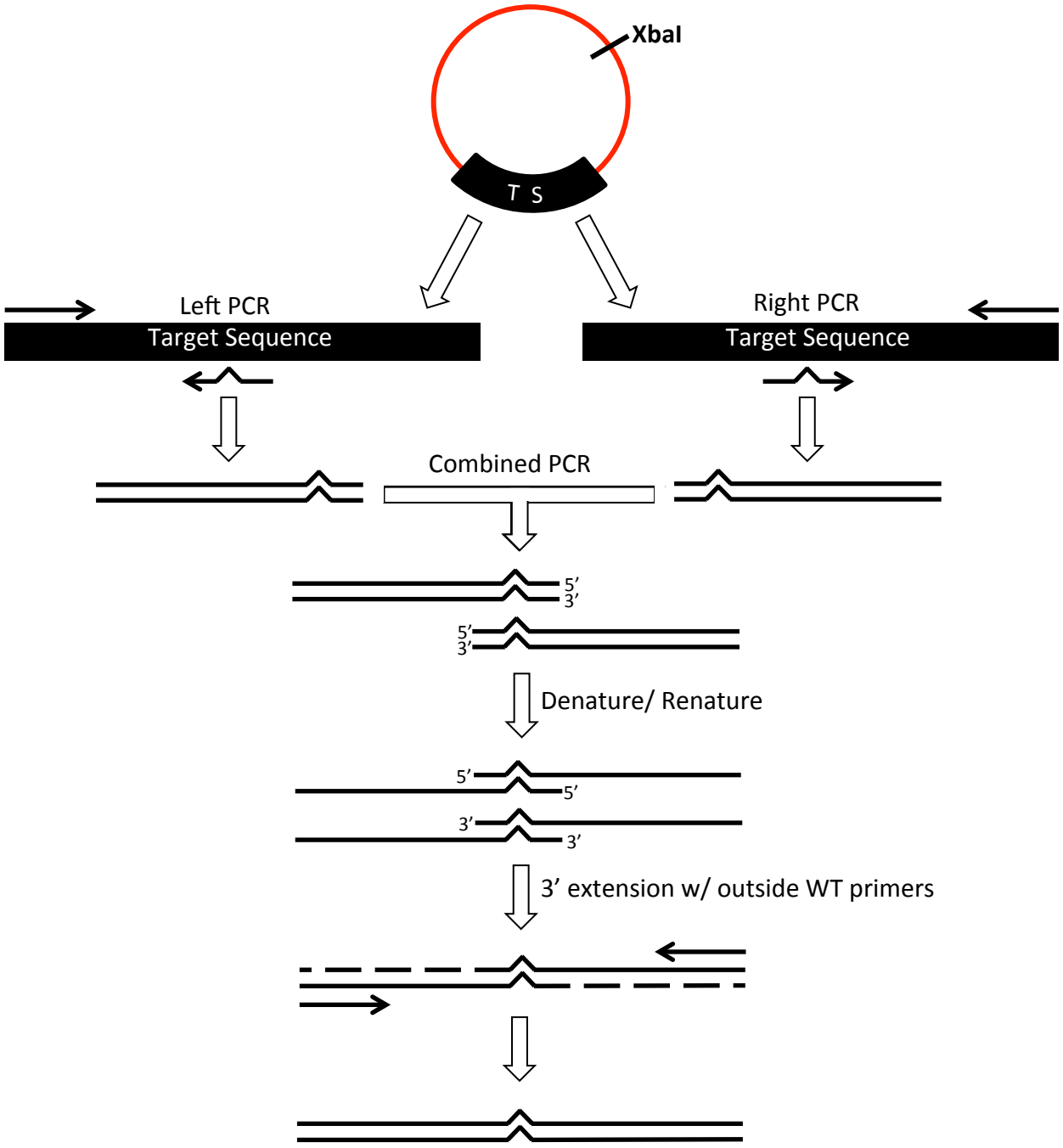


Figure 2. Recombinant PCR. pUCpol was linearized with XbaI and used as a template in two separate reactions designated as the "Left" and "Right" PCR reaction. "Left" PCR reactions involved a forward WT primer and a reverse mutagenic primer. The combination of these two primers in one PCR reaction amplified a small segment of the target gene. The "Right" PCR reaction was the opposite of the "Left" reaction and involved a reverse WT primer and a forward mutagenic primer to create another fragment of the target gene that had a small region of base pairs (bp) that overlapped with the "Left" product. "Left" and "Right" products were combined and used as templates for a final PCR reaction with WT primers that are complementary to sequences that flank the targeted sequence. This final step combines the two segments and forms a product that contains the appropriate target sequence with the desired mutation.

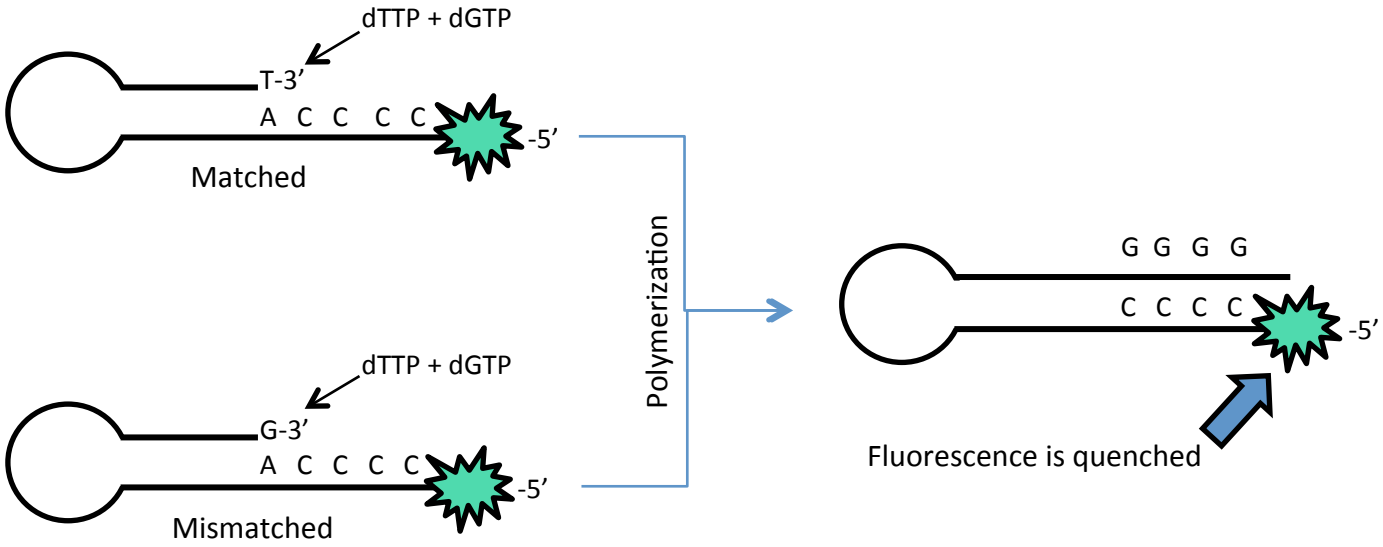


Figure 3. Fluorescence Assay. Probes were designed to fold into a “hairpin” conformation to create either a matched or mismatched base pair at the primer/template interface followed by a cytosine tail that is tagged with the fluorescent marker**.** These probes act as DNA pol substrates and will be used to detect switch behavior. Complete extension of the probe by DNA pol at the 3’- end will result in the quenching of the 5’ fluorescent tag. The quenching of the fluorescent marker depends on the ability for DNA pol to extend beyond the matched or to correct mismatched base pairs and then extend them.

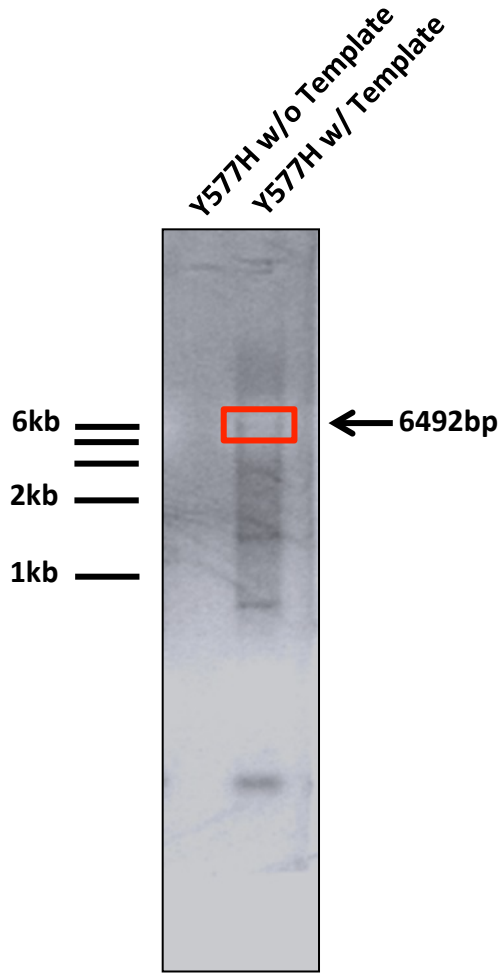


Figure 4. Site-directed Mutagenesis. 10% DMSO was added to the reaction to reduce melting temperatures of the high G+C containing DNA. The template used in the gel was a WT pol gene in pUC19 (pUC19-WT), however, the PCR was equally successful using a wild-type pol in pcDNA. Reactions were carried out with two-step cycles at 94°C for 1 min, followed by at 68°C for 7 min for 30 cycles. Products are separated via electrophoresis through a 0.8% agarose gel. The product in the red box indicates the full-length amplified pUC19-WT plasmid.

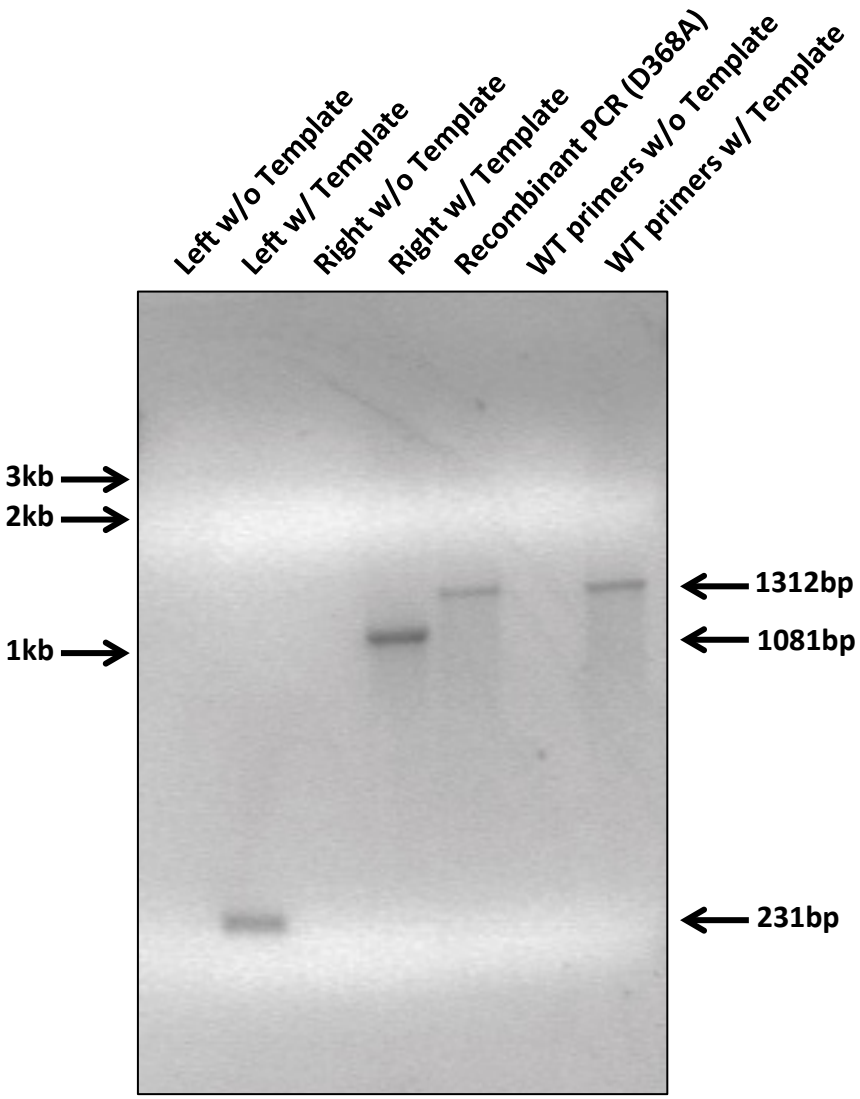


Figure 5. Recombinant PCR Products. Reactions were carried out in three-step cycles with the addition of 10% DMSO at 94°C for 1 min, 62°C for 2 min, and 68°C for 2 min for 30 cycles. Products were separated via electrophoresis through a 1% agarose gel. Refer to Figure 2 for a graphic schematic of this overlapping PCR approach.

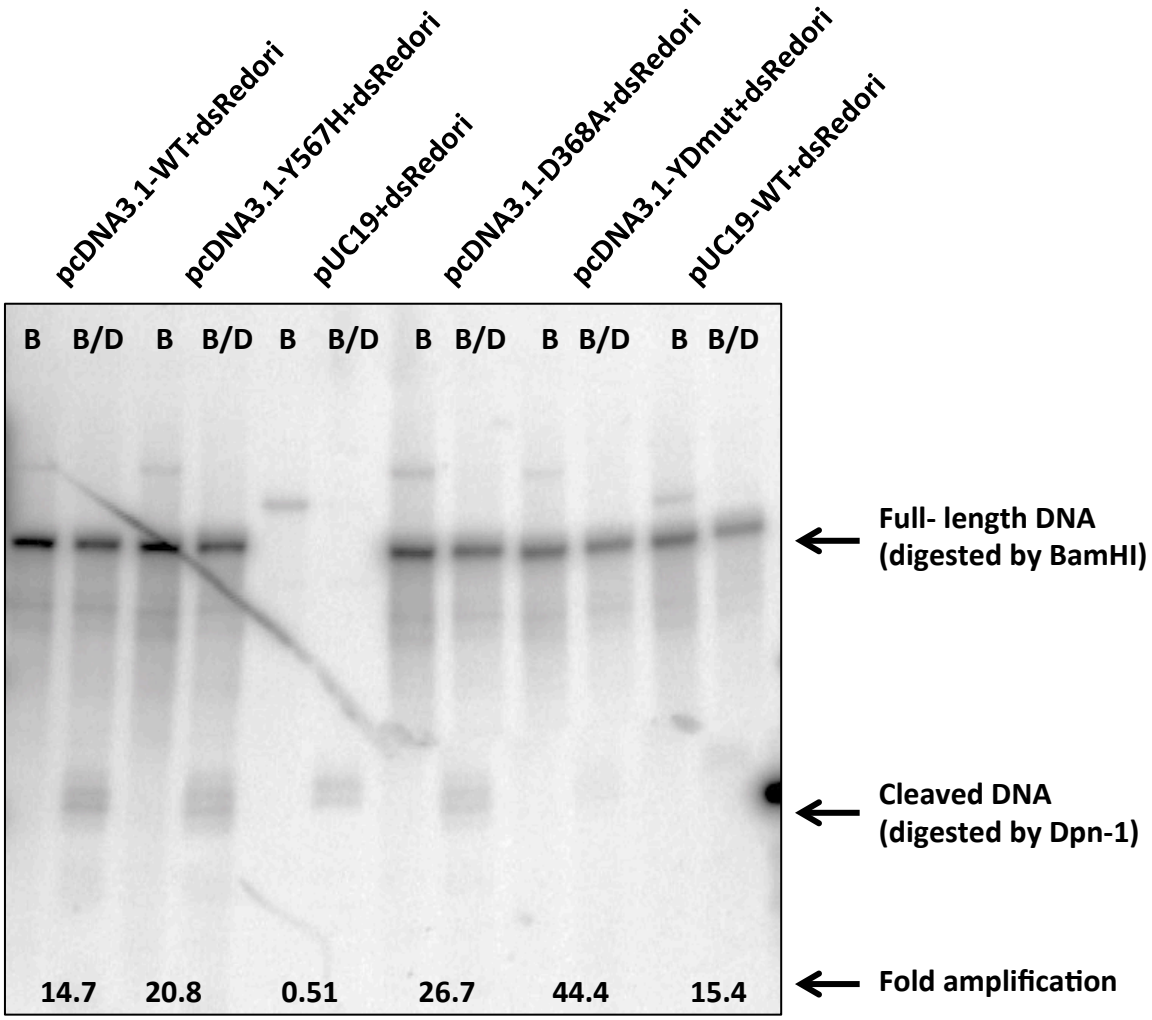


Figure 6. Figure 4. Southern blot of ori-dependent complementation assay of pol Δ mutant HSV-1 . 8×10^5 BHK cells were transfected with pol gene-containing plasmid and an ori-containing gene, dsRed with an ori (dsRedori). Cells were infected with pol gene deleted (Δ) mutant HSV-1 (MOI=5) 24 hours post-transfection. Resulting superinfected cells were lysed and DNA was purified and analyzed through a southern blot. Digested DNA products were subjected to electrophoresis through a 1% agrose gel. DNA samples were treated with BamHI (B) only or with B and Dpn-1 (D) restriction enzymes. Two distinct bands can be seen: the larger band corresponds to full-length plasmid DNA where as the smaller bands are parental DNA digested by Dpn-1. DNA samples treated with B only represent the total amount of DNA present in cells. Fold amplification values for each pol gene were normalized as the amount of radioactivity in the full-length compared to the short fragment in B+D-treated samples.

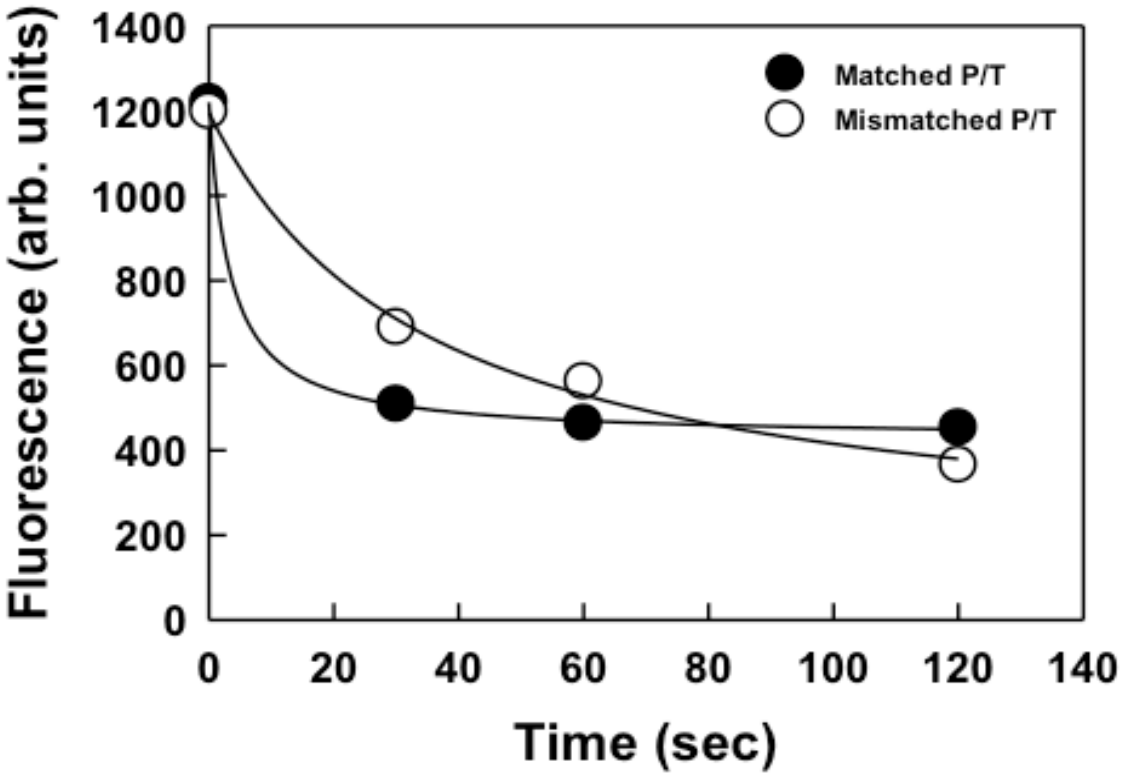


Figure 7. WT pol/UL42 elongating a matched primer/ template (P/T) vs. mismatch P/T in the presence of correcting nucleotides. Matched and mismatched fluorescent hairpin probes (200 nM) were incubated with an excess amount of WT pol/UL42 complex. Reactions were initiated with $MgCl_2$ and stopped at the indicated times by addition of an excess amount of EDTA.

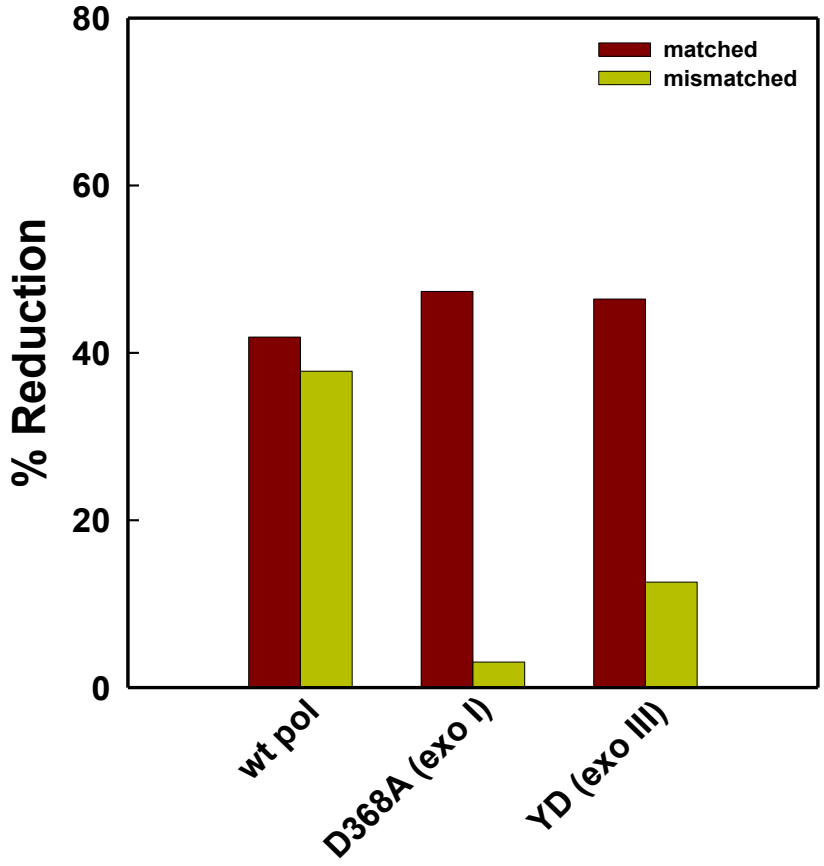


Figure 8. WT pol/UL42 complex vs. exo-deficient pol/UL42 with matched or mismatched P/T. Assays were performed with the same activities of WT and exo-deficient mutant pols as determined by the amount of 3H-TTP incorporated into activated calf thymus DNA template. Probes (200 nM) were treated with the pols for 2 min at 37°C. All of the pols extended matched DNA with similar efficiencies. However, both exo-deficient mutant pols could not excise and repair the mismatch, thus inhibiting the extension of mismatched probe.

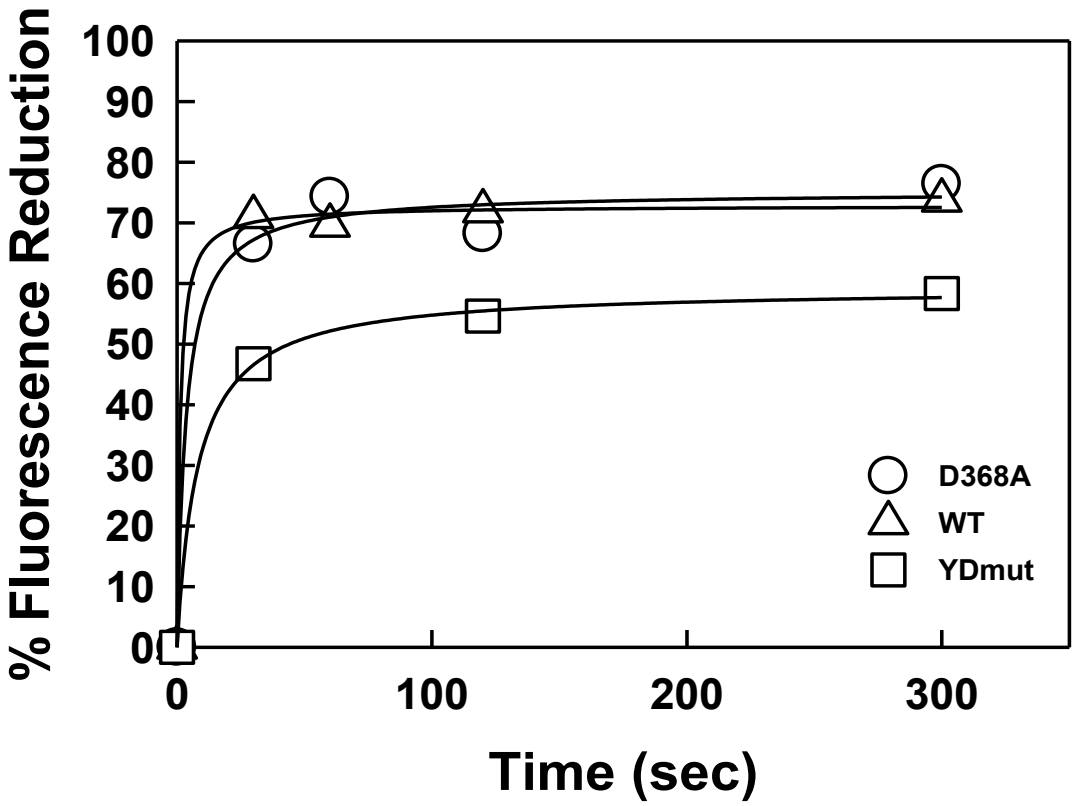


Figure 9. Progression of fluorescence reduction by WT, D368A, and YDmut pol over 5 min. Pre-steady state conditions were simulated by pre-incubation of the enzyme with its DNA substrate in the presence of EDTA. Reactions were initiated by the addition of $MgCl_2$. After a rapid rate of fluorescence reduction that can be seen for all three polymerases, there is little additional reduction most likely due to the exhaustion of DNA substrate by the enzyme .

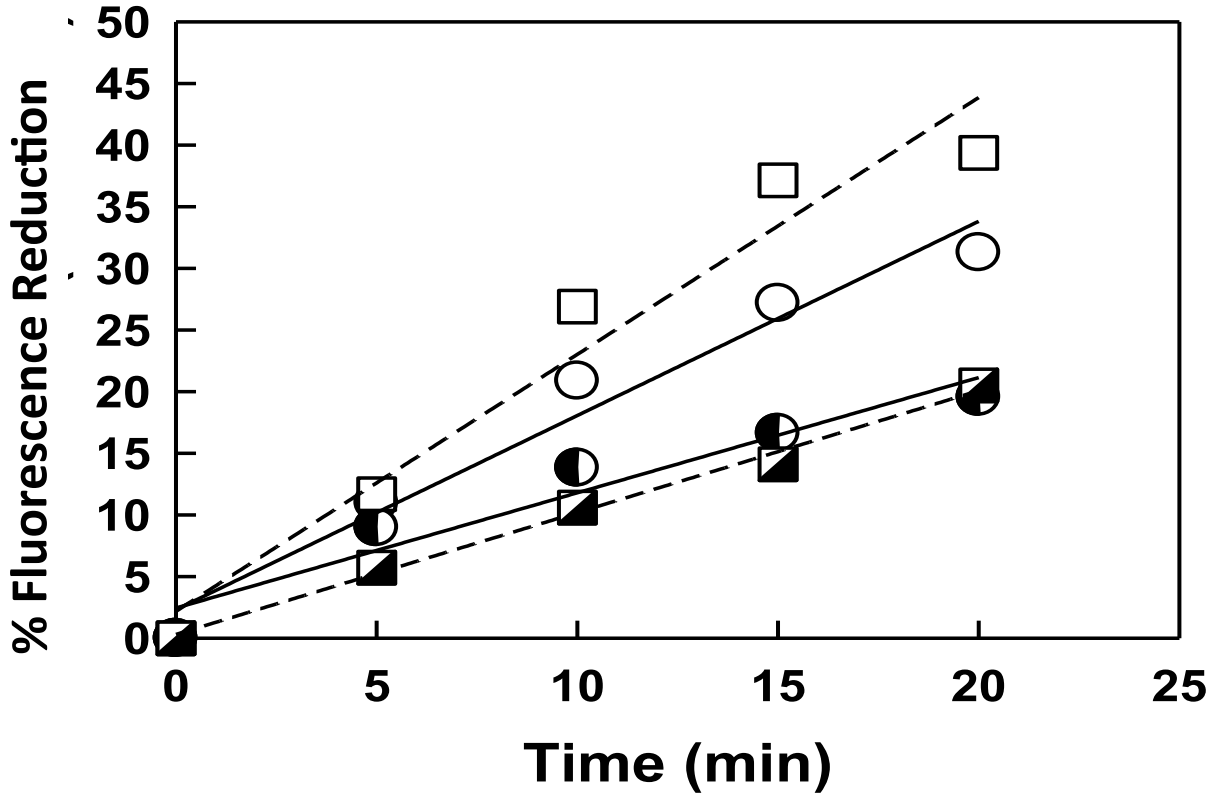
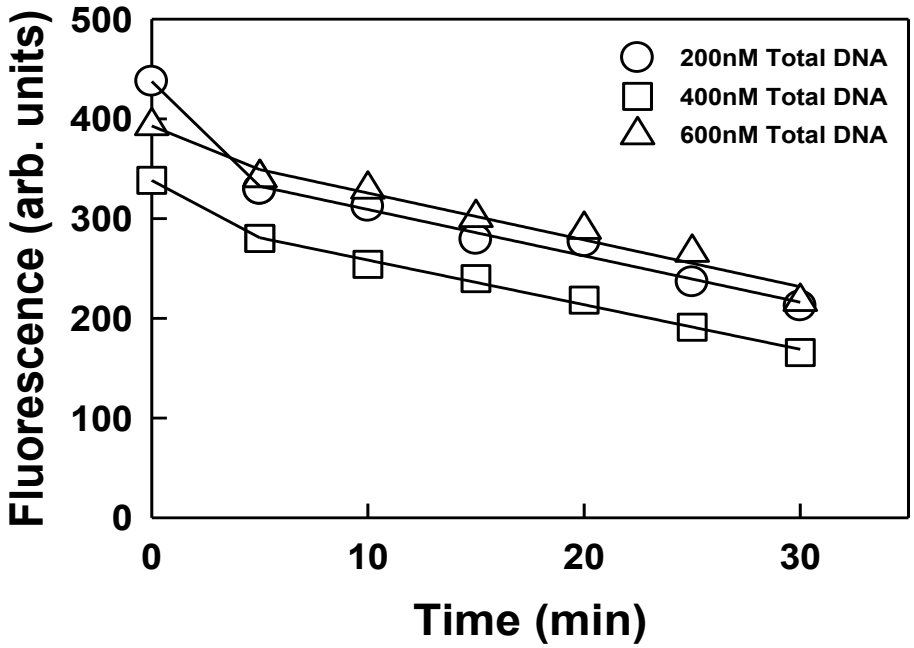


Figure 10. Effect of varying enzyme concentration to matched or mismatched probe. Matched and mismatched probes were separately incubated with two different wild-type pol concentrations (16 units and 32 units).The reaction rate of 32 units of enzyme with a matched probe (-⊡-) was 1.3 times greater than the rate of a 16 unit reaction with a match probe (-⊖-). The reaction rate of 32 units of enzyme with a mismatched probe (-◪-) was similar to that of a 16 unit reaction with a mismatched probe (-◐-).

A.



B.

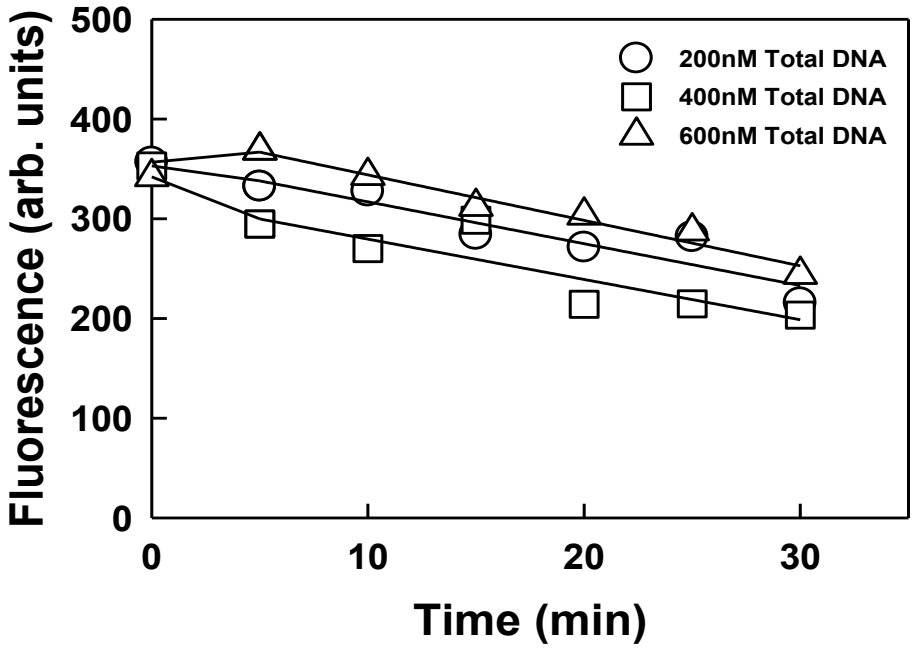


Figure 11. Enzyme response to varying marked probe concentrations. (A). 16 units of enzymatic activity quenching matched fluorescent probes with different total DNA concentrations. Concentrations of fluorescent probes were kept at 200nM with increasing amounts of unlabeled DNA substrates. The rate of fluorescence reduction in the presence of 200nM (◯), 400nM (□), and 600nM (△) were very similar (B). 32 units of enzymatic activity quenching matched fluorescent probes with different dilutions of fluorescent probe. Like that of the 16 units, reactions with 32 units of enzymatic activity had relatively similar rates of reduction regardless of the total amount of DNA present.

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